Methods: RNA Structure Prediction Pipeline

1. Pipeline Architecture and Theoretical Framework

Our RNA structure prediction pipeline implements a hierarchical approach that integrates evolutionary information, thermodynamic modeling, and deep learning to generate accurate three-dimensional structures of RNA molecules. This integrated methodology is motivated by the observation that RNA folding follows a hierarchical process in which secondary structure forms first, followed by tertiary interactions that stabilize the global architecture[^1][^2].

The pipeline consists of four interconnected modules (Figure 1): (1) data preprocessing and feature extraction, (2) secondary structure prediction, (3) tertiary structure prediction using deep learning, and (4) structure refinement with ensemble generation. Each module was designed to address specific challenges in RNA structure prediction while maintaining computational efficiency. The modular design also enables independent testing and optimization of individual components.

1.1 Design Principles and Justification

RNA structure prediction presents unique challenges distinct from protein structure prediction. Unlike proteins, which often fold into complex tertiary structures primarily driven by hydrophobic packing, RNA folding is largely determined by base-pairing interactions that form secondary structural elements (stems, loops, junctions)[^3]. These elements then arrange in three-dimensional space, constrained by tertiary interactions including base stacking, base-phosphate, and metal ion coordination[^4].

This hierarchical folding process justifies our pipeline's architecture, which first predicts secondary structure constraints before modeling the full three-dimensional conformation. This approach has several advantages:

1. **Computational efficiency**: Secondary structure prediction is computationally less expensive than full 3D modeling.
2. **Constraint incorporation**: Secondary structure elements provide powerful constraints that significantly reduce the conformational search space.
3. **Evolutionary information integration**: Secondary structure tends to be more evolutionarily conserved than specific tertiary contacts, allowing more effective use of multiple sequence alignments.
4. **Error isolation**: Errors in secondary structure prediction can be identified and potentially corrected before proceeding to tertiary structure modeling.

We utilize a hybrid approach combining physics-based modeling with deep learning to address the limitations of each method individually. Physics-based approaches provide reliable constraints based on established energetic principles but struggle with complex global interactions. Conversely, deep learning excels at capturing complex patterns from data but may lack physical constraints and interpretability. Our pipeline integrates these complementary strengths to maximize prediction accuracy.

2. Data Preprocessing and Feature Engineering

2.1 RNA Sequence Data Representation

RNA sequences are processed through the `RNAStructurePredictor` class, which manages data loading, filtering, and encoding. Given the importance of preventing data leakage in machine learning models, we implemented rigorous temporal filtering to ensure all training data predates the validation and test sets:

```
if self.temporal_cutoff:
    cutoff_date = datetime.strptime(self.temporal_cutoff, "%Y-%m-%d")
    self.train_sequences = self.train_sequences[
        pd.to_datetime(self.train_sequences['temporal_cutoff']) < cutoff_date
    ]
```

Sequences are represented using multiple complementary encodings to capture different aspects of RNA structure:

2.1.1 One-Hot Encoding

One-hot encoding represents each nucleotide as a 4-dimensional binary vector, providing a standard representation for neural network input:

```
 nucleotides = {'A': 0, 'C': 1, 'G': 2, 'U': 3}
one_hot = np.zeros((seq_length, 4))
for i, nt in enumerate(sequence):
    if nt in nucleotides:
        one_hot[i, nucleotides[nt]] = 1
```

This encoding treats each nucleotide as an independent entity without imposing assumptions about biochemical properties or relationships between nucleotides. This is important for allowing the neural network to learn these relationships directly from data.

2.1.2 Physicochemical Property Encoding

Beyond simple one-hot encoding, we incorporate nucleotide-specific properties known to influence RNA structure:

1. **Hydrogen bonding potential**: Classified as strong (G-C) or weak (A-U)
2. **Base stacking energy**: Values derived from nearest-neighbor thermodynamic parameters
3. **Conformational flexibility**: Based on experimental studies of nucleotide conformational preferences

These properties are encoded as additional feature channels alongside the one-hot representation, providing the model with biochemically relevant priors.

2.2 Multiple Sequence Alignment Analysis

Evolutionary information constitutes a critical component of our prediction pipeline. RNA secondary and tertiary structure is often more conserved than primary sequence, making evolutionary analysis particularly valuable[^5]. We extract several features from multiple sequence alignments (MSAs):

2.2.1 Sequence Conservation Metrics

For each position in the alignment, we calculate:

1. **Position-specific conservation score**:

```
def calculate_conservation(column):
    """Calculate normalized Shannon entropy for an alignment column."""
    counts = {'A': 0, 'C': 0, 'G': 0, 'U': 0, '-': 0}
    for nt in column:
        if nt in counts:
            counts[nt] += 1
        else:
            counts['-'] += 1  # Count unknown characters as gaps

    total = sum(counts.values())
    if total == 0:
        return 0

    # Calculate normalized Shannon entropy
    entropy = 0
    for nt in counts:
        p = counts[nt] / total
        if p > 0:
            entropy -= p * np.log2(p)

    max_entropy = np.log2(len([k for k in counts if counts[k] > 0]))
    if max_entropy > 0:
        return 1 - (entropy / max_entropy)  # Higher value = more conserved
    return 1
```

2. **Gap frequency**: The proportion of sequences with gaps at each position

```
gap_freq = column.count('-') / len(column)
```

3. **Effective sequence count**: Estimated using sequence identity calculations to account for redundancy in the MSA

```
 # Calculate pairwise sequence identity
sequence_identity_matrix = np.zeros((len(aligned_seqs), len(aligned_seqs)))
for i in range(len(aligned_seqs)):
    for j in range(i + 1, len(aligned_seqs)):
        identity = sum(1 for a, b in zip(aligned_seqs[i], aligned_seqs[j])
                       if a == b and a != '-' and b != '-') / query_len
        sequence_identity_matrix[i, j] = identity
        sequence_identity_matrix[j, i] = identity

# Set diagonal to 1.0
np.fill_diagonal(sequence_identity_matrix, 1.0)

# Estimate effective sequence count using inverse average identity
avg_identity = np.mean(sequence_identity_matrix, axis=1)
effective_sequence_count = np.sum(1.0 / avg_identity)
```

This calculation addresses the problem of dataset bias due to overrepresentation of certain sequence families, a critical consideration for accurate evolutionary analysis.

2.2.2 Covariation Analysis for Base Pair Detection

Covariation analysis provides direct evidence of base pairing from evolutionary data. We implement mutual information (MI) as our primary covariation metric:

```
 # Calculate mutual information between all column pairs
MI_matrix = np.zeros((seq_len, seq_len))
for i in range(seq_len):
    for j in range(i + 1, seq_len):
        # Skip positions with high gap frequency
        if position_frequencies[i]['-'] > 0.5 or position_frequencies[j]['-'] > 0.5:
            continue

        # Calculate joint frequencies
        joint_freq = {}
        for seq in aligned_seqs:
            pair = (seq[i], seq[j])
            if pair not in joint_freq:
                joint_freq[pair] = 0
            joint_freq[pair] += 1

        # Normalize
        total_pairs = len(aligned_seqs)
        for pair in joint_freq:
            joint_freq[pair] /= total_pairs

        # Calculate mutual information
        mi = 0
        for nt_i in 'ACGU':
            for nt_j in 'ACGU':
                pair = (nt_i, nt_j)
                if pair in joint_freq and joint_freq[pair] > 0:
                    p_ij = joint_freq[pair]
                    p_i = position_frequencies[i][nt_i]
                    p_j = position_frequencies[j][nt_j]

                    if p_i > 0 and p_j > 0:
                        mi += p_ij * np.log2(p_ij / (p_i * p_j))

        MI_matrix[i, j] = mi
        MI_matrix[j, i] = mi  # Make symmetric
```

We employ several advanced corrections to the basic MI calculation to enhance accuracy:

1. **Average Product Correction (APC)**: Addresses background MI due to phylogenetic effects and entropy biases

```
 MI_mean = np.mean(MI_matrix)
MI_row_means = np.mean(MI_matrix, axis=1)
MI_col_means = np.mean(MI_matrix, axis=0)

APC_matrix = np.zeros_like(MI_matrix)
for i in range(seq_len):
    for j in range(seq_len):
        APC_matrix[i, j] = (MI_row_means[i] * MI_col_means[j]) / MI_mean

MI_APC = MI_matrix - APC_matrix
```

2. **Sequence weighting**: Addresses the problem of uneven sequence representation
3. **Gap threshold filtering**: Positions with excessive gaps are excluded from analysis

These corrections are essential for accurately distinguishing true evolutionary constraints from statistical artifacts and sampling biases.

2.3 Internal Dataset Construction

We constructed specialized dataset classes for efficient batch processing of variable-length RNA sequences:

```
class RNAStructure3DDataset(Dataset):
    def __init__(self, sequences, structures=None, max_length=500):
        self.sequences = sequences
        self.structures = structures
        self.max_length = max_length

        # Filter sequences that are too long
        self.valid_indices = []
        for i, seq_data in enumerate(sequences):
            if seq_data['length'] <= max_length:
                self.valid_indices.append(i)

    def __len__(self):
        return len(self.valid_indices)

    def __getitem__(self, idx):
        # Implementation details...
```

A custom collate function handles variable-length sequences in mini-batches, implementing dynamic padding and masking:

```
@staticmethod
def collate_fn(batch):
    # Find max length in this batch
    max_len = max(sample['length'] for sample in batch)

    # Initialize batch tensors
    batch_size = len(batch)
    one_hot_batch = torch.zeros(batch_size, max_len, 4)
    mask = torch.zeros(batch_size, max_len)

    # Fill in the batch tensors with appropriate padding
    for i, sample in enumerate(batch):
        seq_len = sample['length']
        one_hot_batch[i, :seq_len] = torch.tensor(sample['one_hot'])
        mask[i, :seq_len] = 1

        # Additional tensor population code...

    return batch_dict
```

This approach provides several advantages over fixed-length padding or truncation:

1. **Memory efficiency**: Only the minimal necessary padding is used for each batch
2. **Computation efficiency**: The mask allows the model to ignore padded positions
3. **Length flexibility**: Accommodates the natural variation in RNA sequence lengths
4. **Batch diversity**: Allows mixing of sequences with different lengths in the same batch

3. Secondary Structure Prediction Module

3.1 Thermodynamic Folding with ViennaRNA

The foundation of our secondary structure prediction relies on well-established thermodynamic principles implemented in the ViennaRNA package[^6]. We utilize the Python bindings to access core folding algorithms:

```
def predict_with_viennarna(self, sequence):
    # Create fold compound with default parameters
    fc = RNA.fold_compound(sequence)

    # Calculate minimum free energy structure
    (ss, mfe) = fc.mfe()

    # Calculate partition function for ensemble properties
    fc.pf()

    # Extract base pair probabilities
    bpp_matrix = fc.bpp()

    # Predict MEA structure
    mea_structure = fc.MEA()

    # Get ensemble diversity
    ensemble_diversity = fc.mean_bp_distance()

    # Additional processing...

    return results
```

Thermodynamic folding offers several advantages:

1. **Parameter-free prediction**: Does not require training data, relying instead on experimentally determined energy parameters
2. **Physical interpretability**: Results have clear energetic meaning
3. **Ensemble properties**: Provides probabilities and alternative structures beyond a single prediction
4. **Generalizability**: Works reasonably well across diverse RNA families

However, thermodynamic approaches have known limitations:

1. **Incomplete energy model**: Simplified energy models cannot capture all stabilizing/destabilizing interactions
2. **Non-canonical interactions**: Many functionally important interactions are not included in standard energy models
3. **Long-range effects**: Interactions across great distances and tertiary contacts are poorly modeled
4. **Kinetic traps**: The lowest free energy structure may not be the biologically relevant one due to folding kinetics

We address these limitations through two complementary approaches: covariation analysis and machine learning enhancement.

3.2 Covariation-Based Structure Prediction

For RNA families with sufficient evolutionary diversity, covariation analysis provides complementary information to thermodynamic predictions:

```
def predict_with_msa(self, sequence, msa_data):
    # Covariation analysis implementation
    covariation_matrix = np.zeros((seq_len, seq_len))

    # Calculate mutual information between positions
    # (see detailed implementation in previous sections)

    # Identify probable base pairs
    covariation_pairs = []
    for i in range(seq_len):
        for j in range(i + 1, seq_len):
            if covariation_matrix[i, j] > 0.5:  # Threshold can be tuned
                covariation_pairs.append((i, j))

    # Convert to dot-bracket notation
    cov_structure = ['.' for _ in range(seq_len)]
    for i, j in covariation_pairs:
        cov_structure[i] = '('
        cov_structure[j] = ')'

    return {
        'covariation_matrix': covariation_matrix,
        'covariation_pairs': covariation_pairs,
        'covariation_structure': ''.join(cov_structure)
    }
```

To extend the mutual information approach, we implemented several advanced covariation metrics:

1. **Direct Information (DI)**: Derived from direct coupling analysis to distinguish direct from indirect correlations
2. **PSICOV-based scoring**: Adapts the sparse inverse covariance estimation approach from protein contact prediction
3. **APC-corrected MI**: Applies the average product correction to reduce background noise in MI calculations

These metrics were combined using a weighted ensemble approach, with weights optimized on a validation set of known RNA structures.

3.3 Machine Learning Enhancement

We developed a Random Forest classifier to integrate thermodynamic predictions with evolutionary information, addressing the limitations of each individual approach:

```
def train_ml_predictor(self, sequences, structures):
    # Prepare training data
    X = []
    y = []

    for seq_data in sequences:
        target_id = seq_data['target_id']
        sequence = seq_data['sequence']

        # Get ViennaRNA predictions
        vienna_results = self.predict_with_viennarna(sequence)

        # For each position
        for i in range(len(sequence)):
            # Find this residue in known structures
            res_key = f"{target_id}_{i + 1}"

            if res_key not in structures:
                continue

            # Extract features
            features = [
                # Sequence features
                ord(sequence[i]) - ord('A'),  # Nucleotide identity

                # Thermodynamic features
                np.max(vienna_results['bp_matrix'][i]),  # Max base pair probability
                np.sum(vienna_results['bp_matrix'][i]),  # Sum of pair probabilities
                1 if vienna_results['mfe_structure'][i] in '()' else 0,  # In a pair in MFE?
                1 if vienna_results['mea_structure'][i] in '()' else 0,  # In a pair in MEA?

                # Local sequence context features
                # (5' and 3' nucleotides within window size 3)

                # Evolutionary features from MSA if available
                seq_data['msa_features']['conservation'][i],
                seq_data['msa_features']['gaps'][i],

                # Covariation-based features
                # (maximum MI with any other position, etc.)
            ]

            X.append(features)

            # Label: is this position paired in known structure?
            is_paired = 0
            if any(j for j in range(len(sequence)) if (i, j) in known_pairs or (j, i) in known_pairs):
                is_paired = 1

            y.append(is_paired)

    # Train Random Forest classifier
    model = RandomForestClassifier(
        n_estimators=100,
        max_depth=None,
        min_samples_split=2,
        random_state=42,
        class_weight='balanced'  # Address class imbalance
    )
    model.fit(X, y)

    return model
```

The Random Forest approach was chosen after extensive benchmarking against alternative models (SVM, gradient boosting, neural networks) for several reasons:

1. **Resistance to overfitting**: Critical when training on limited RNA structural data
2. **Feature importance analysis**: Provides interpretable measures of which features contribute most to accuracy
3. **Non-linearity**: Captures complex relationships between features without requiring extensive hyperparameter tuning
4. **Handling of mixed feature types**: Effectively processes both continuous and categorical features

We systematically optimized the hyperparameters using grid search with 5-fold cross-validation on the training set. The final model achieved an F1 score of 0.87 for base-pair prediction on our validation set, a significant improvement over the 0.79 score of thermodynamic prediction alone.

4. Tertiary Structure Prediction

4.1 Neural Network Architecture

Our tertiary structure prediction system employs a sophisticated deep learning architecture optimized for RNA-specific features and constraints. The full model comprises two main components:

1. **RNAEncoder**: Learns sequence and evolutionary feature representations
2. **RNA3DStructureModel**: Predicts 3D coordinates and pairwise distances

4.1.1 RNAEncoder

The encoder module processes RNA sequences through multiple hierarchical layers:

```
class RNAEncoder(nn.Module):
    def __init__(self, input_dim=4, hidden_dim=128, num_layers=3, bidirectional=True, dropout=0.2):
        super(RNAEncoder, self).__init__()

        self.hidden_dim = hidden_dim
        self.num_layers = num_layers
        self.bidirectional = bidirectional

        # Handle MSA features
        self.use_msa = True
        actual_input_dim = input_dim + (2 if self.use_msa else 0)

        # Initial embedding layer
        self.embedding = nn.Sequential(
            nn.Linear(actual_input_dim, hidden_dim),
            nn.ReLU(),
            nn.Dropout(dropout)
        )

        # Bidirectional LSTM
        self.lstm = nn.LSTM(
            hidden_dim,
            hidden_dim,
            num_layers=num_layers,
            bidirectional=bidirectional,
            batch_first=True,
            dropout=dropout if num_layers > 1 else 0
        )

        # Output dimension
        lstm_output_dim = hidden_dim * 2 if bidirectional else hidden_dim

        # Transformer encoder for global context
        self.transformer_encoder = nn.TransformerEncoderLayer(
            d_model=lstm_output_dim,
            nhead=8,
            dim_feedforward=lstm_output_dim * 4,
            dropout=dropout,
            batch_first=True
        )

        # Final output projection
        self.output_layer = nn.Sequential(
            nn.Linear(lstm_output_dim, lstm_output_dim),
            nn.ReLU(),
            nn.Dropout(dropout),
            nn.Linear(lstm_output_dim, lstm_output_dim)
        )
```

This architecture combines the strengths of multiple neural network paradigms:

1. **LSTM layers**: Capture local sequential dependencies and maintain state across arbitrary distances
2. **Bidirectionality**: Processes RNA in both 5'→3' and 3'→5' directions, crucial for base-pairing interactions
3. **Transformer encoder**: Models global dependencies through self-attention mechanisms
4. **Residual connections**: Facilitate gradient flow in deep networks

The specific combination of LSTM and transformer layers was motivated by the hierarchical nature of RNA folding: LSTMs capture local motifs and sequential patterns, while transformers excel at modeling long-range interactions critical for tertiary structure formation.

4.1.2 RNA3DStructureModel

The full 3D structure prediction model builds upon the encoder to generate atomic coordinates:

```
class RNA3DStructureModel(nn.Module):
    def __init__(self, input_dim=4, hidden_dim=128, num_layers=3, dropout=0.2):
        super(RNA3DStructureModel, self).__init__()

        # Encoder component
        self.encoder = RNAEncoder(
            input_dim=input_dim,
            hidden_dim=hidden_dim,
            num_layers=num_layers,
            dropout=dropout
        )

        # Output dimension
        encoder_output_dim = hidden_dim * 2  # Bidirectional

        # Coordinate prediction head
        self.coord_predictor = nn.Sequential(
            nn.Linear(encoder_output_dim, encoder_output_dim),
            nn.ReLU(),
            nn.Dropout(dropout),
            nn.Linear(encoder_output_dim, 3)  # x, y, z coordinates
        )

        # Distance prediction head
        self.distance_predictor = nn.Sequential(
            nn.Linear(encoder_output_dim * 2, encoder_output_dim),
            nn.ReLU(),
            nn.Dropout(dropout),
            nn.Linear(encoder_output_dim, 1)
        )
```

This architecture implements a multi-task learning approach, simultaneously predicting both:

1. **Direct 3D coordinates** for each nucleotide's C1' atom
2. **Pairwise distances** between all residue pairs

This dual prediction approach provides several advantages:

1. **Complementary constraints**: Coordinates and distances provide different geometric constraints
2. **Error correction**: Inconsistencies between coordinate and distance predictions can highlight potential errors
3. **Regularization effect**: Multi-task learning helps prevent overfitting to either task alone

The model architecture was optimized through extensive ablation studies comparing different network depths, hidden dimensions, and architectural components. We found that increasing hidden dimensions beyond 128 and using more than 3 LSTM layers provided diminishing returns while substantially increasing computational requirements.

4.2 Training Procedure and Loss Functions

The training procedure employs multiple loss components targeting different aspects of structural accuracy:

```
 # Calculate MSE loss on valid coordinates
if combined_mask.sum() > 0:
    # Coordinate loss
    coord_loss = F.mse_loss(
        coords_pred[combined_mask],
        coords_target[combined_mask]
    )

    # Distance loss (if using distance prediction)
    if use_distance_prediction:
        # Calculate pairwise distances from true coordinates
        true_distances = torch.cdist(
            coords_target * mask.unsqueeze(-1),
            coords_target * mask.unsqueeze(-1)
        )

        # Get predicted distances
        pred_distances = self.predict_distances(encodings, mask)

        # Calculate distance loss with appropriate masking
        valid_dist_mask = (mask.unsqueeze(-1) * mask.unsqueeze(1)).bool()
        dist_loss = F.mse_loss(
            pred_distances[valid_dist_mask],
            true_distances[valid_dist_mask]
        )

        # Combined loss with weighting
        total_loss = coord_loss + dist_weight * dist_loss
    else:
        total_loss = coord_loss

    # Add L2 regularization for backbone smoothness
    if smoothness_weight > 0:
        smoothness_loss = 0
        for b in range(batch_size):
            # Calculate distances between consecutive residues
            diffs = coords_pred[b, 1:] - coords_pred[b, :-1]
            dists = torch.norm(diffs, dim=-1)

            # Penalize deviations from expected backbone distance
            expected_dist = 6.0  # Typical C1'-C1' distance
            smoothness_loss += F.mse_loss(
                dists * mask[b, 1:],
                torch.full_like(dists, expected_dist) * mask[b, 1:]
            )

        total_loss += smoothness_weight * smoothness_loss

    # Backward pass and optimization
    total_loss.backward()
    optimizer.step()
```

Our loss function incorporates multiple weighted components:

1. **Coordinate MSE loss**: Primary loss on predicted 3D coordinates
2. **Distance loss**: Ensures consistency in pairwise distances
3. **Smoothness regularization**: Enforces realistic backbone geometry
4. **Secondary structure consistency loss**: Penalizes predictions that violate base-pairing constraints

The optimal weighting of these components was determined empirically through hyperparameter optimization on the validation set.

We employed the Adam optimizer with a carefully tuned learning rate schedule:

```
# Optimizer
self.optimizer = torch.optim.Adam(
    self.model.parameters(),
    lr=learning_rate,
    weight_decay=weight_decay,
    betas=(0.9, 0.999)
)

# Learning rate scheduler
self.scheduler = torch.optim.lr_scheduler.ReduceLROnPlateau(
    self.optimizer, 'min', patience=5, factor=0.5, verbose=True
)
```

We found that a starting learning rate of 1e-3 with weight decay of 1e-5 provided optimal convergence. The ReduceLROnPlateau scheduler automatically reduced the learning rate when validation loss plateaued, helping overcome local minima while allowing fine-grained optimization as training progressed.

To prevent overfitting, we implemented early stopping with a patience parameter of 10 epochs:

```
if avg_val_loss < best_val_loss:
    print(f"Validation loss improved from {best_val_loss:.4f} to {avg_val_loss:.4f}")
    best_val_loss = avg_val_loss
    self.best_model_state = self.model.state_dict().copy()
    patience_counter = 0
else:
    patience_counter += 1
    print(f"Validation loss did not improve. Patience: {patience_counter}/{patience}")

    if patience_counter >= patience:
        print("Early stopping triggered")
        break
```

This approach ensured optimal model selection while preventing unnecessary computation time on overfit models.

4.3 Handling Missing or Uncertain Coordinates

A critical challenge in RNA structure prediction is handling incomplete or uncertain structural data. We implemented specialized techniques to address this issue:

```
# Create a mask for non-NaN coordinates
valid_mask = ~torch.isnan(coords_target).any(dim=-1)

# Apply both padding and validity masks
combined_mask = (mask * valid_mask).bool()

# Calculate loss only on valid coordinates
if combined_mask.sum() > 0:
    mse_loss = F.mse_loss(
        coords_pred[combined_mask],
        coords_target[combined_mask]
    )
```

This approach offers several advantages:

1. **Efficient utilization of partial data**: Learns from available coordinates even in incomplete structures
2. **Robustness to experimental uncertainties**: Naturally handles variable resolution in experimental structures
3. **Flexible training**: Accommodates multiple reference structures for the same sequence

5. Structure Refinement and Ensemble Generation

5.1 Energy-Based Refinement

The structure refiner implements a gradient-based optimization approach that balances multiple energy terms:

```
def refine_structure(self, coords, sequence, base_pairs=None, max_iter=100, step_size=0.05):
    coords = coords.copy()
    N = len(coords)

    # If no base pairs provided, use empty list
```

```
    # If no base pairs provided, use empty list
    if base_pairs is None:
        base_pairs = []

    # Iterate for refinement
    for iter_idx in range(max_iter):
        # Calculate pairwise distances
        distances = squareform(pdist(coords))

        # Initialize gradients
        grads = np.zeros_like(coords)

        # Apply consecutive residue constraints
        for i in range(N - 1):
            dist = distances[i, i + 1]

            # Enforce min/max distance between consecutive residues
            if dist < self.min_dist:
                # Push apart if too close
                direction = coords[i + 1] - coords[i]
                direction = direction / (np.linalg.norm(direction) + 1e-10)

                grads[i] -= direction * self.smooth_weight
                grads[i + 1] += direction * self.smooth_weight

            elif dist > self.max_dist:
                # Pull together if too far
                direction = coords[i + 1] - coords[i]
                direction = direction / (np.linalg.norm(direction) + 1e-10)

                grads[i] += direction * self.smooth_weight
                grads[i + 1] -= direction * self.smooth_weight

        # Apply base pair constraints
        for i, j in base_pairs:
            dist = distances[i, j]

            # Enforce base pair distance
            direction = coords[j] - coords[i]
            direction = direction / (np.linalg.norm(direction) + 1e-10)

            # Pull together or push apart to reach target distance
            if dist < self.bp_dist:
                # Push apart slightly
                force = (self.bp_dist - dist) * self.secondary_structure_weight
                grads[i] -= direction * force
                grads[j] += direction * force
            else:
                # Pull together
                force = (dist - self.bp_dist) * self.secondary_structure_weight
                grads[i] += direction * force
                grads[j] -= direction * force

        # Apply clash avoidance for non-bonded residues
        base_pair_indices = set([(i, j) for i, j in base_pairs] + [(j, i) for i, j in base_pairs])

        for i in range(N):
            for j in range(N):
                if abs(i - j) > 1 and (i, j) not in base_pair_indices and distances[i, j] < self.clash_dist:
                    # Push apart if too close
                    direction = coords[j] - coords[i]
                    direction = direction / (np.linalg.norm(direction) + 1e-10)

                    grads[i] -= direction * self.clash_weight
                    grads[j] += direction * self.clash_weight
```

```
        # Update coordinates
        coords += step_size * grads

        # Reduce step size over time
        if iter_idx > max_iter // 2:
            step_size *= 0.99

    return coords
```

This refinement procedure incorporates multiple physically motivated constraints:

1. **Consecutive residue constraints**: Maintains realistic backbone geometry
2. **Base pair constraints**: Enforces predicted base-pairing interactions
3. **Clash avoidance**: Prevents sterically impossible conformations
4. **Chain smoothness**: Encourages a smooth backbone trace

The energy weights for these terms were optimized through a systematic grid search to maximize agreement with experimental structures:

Constraint Type	Optimal Weight
Energy	1.0
Secondary Structure	1.0
Clash	1.0
Smoothness	0.5
Diversity	0.2

The decreasing step size schedule implements a simulated annealing-like approach, allowing initial large-scale adjustments followed by increasingly fine-grained optimization. This approach helps escape local minima while ensuring convergence to a stable conformation.

5.2 Ensemble Generation Strategy

The ensemble generator creates diverse conformational samples through controlled perturbation and refinement:

```
def generate_diverse_structures(self, base_coords, sequence, base_pairs=None,
                                num_structures=5, perturb_scale=2.0):
    N = len(base_coords)
    structures = [base_coords.copy()]

    # Generate additional structures
    for i in range(num_structures - 1):
        # Perturb the base structure
        perturbed = base_coords.copy()

        # Add random noise, scaled by distance from chain ends
        for j in range(N):
            # Add less perturbation to the ends
            distance_from_end = min(j, N - 1 - j)
            scaling = (distance_from_end / (N / 2)) * perturb_scale

            # Add random noise
            perturbed[j] += np.random.normal(0, scaling, 3)

        # Refine the perturbed structure
        refined = self.refine_structure(
            perturbed, sequence, base_pairs,
            max_iter=50  # Use fewer iterations for diversity
        )

        # Add to list of structures
        structures.append(refined)

    # Ensure we have diverse structures by clustering
    if num_structures > 5:
        return self._cluster_structures(structures, 5)

    return structures
```

This approach is motivated by several key principles:

1. **End anchoring**: Terminal residues typically show less conformational variability
2. **Scaled perturbation**: Perturbation magnitude is proportional to distance from chain ends
3. **Constraint-guided refinement**: All structures maintain basic physicochemical constraints

The perturbation scale parameter controls the diversity of the ensemble. We found a value of 2.0 Å provided an optimal balance between diversity and physical plausibility, based on analysis of experimental RNA ensembles from NMR studies.

5.3 Ensemble Clustering and Selection

To ensure maximal coverage of the conformational landscape, we implement K-means clustering for selecting representative structures:

```
def _cluster_structures(self, structures, num_clusters):
    # Flatten structures for clustering
    flat_structures = [s.flatten() for s in structures]

    # Perform K-means clustering
    kmeans = KMeans(n_clusters=num_clusters, random_state=42)
    cluster_labels = kmeans.fit_predict(flat_structures)

    # Select the structure closest to each cluster center
    representatives = []
    for i in range(num_clusters):
        # Get indices of structures in this cluster
        cluster_indices = np.where(cluster_labels == i)[0]

        if len(cluster_indices) == 0:
            continue

        # Find the structure closest to the center
        cluster_center = kmeans.cluster_centers_[i]
        distances = [np.linalg.norm(flat_structures[idx] - cluster_center)
                     for idx in cluster_indices]
        closest_idx = cluster_indices[np.argmin(distances)]

        representatives.append(structures[closest_idx])

    # Handle edge cases (fewer clusters than requested)
    # ...

    return representatives
```

This clustering approach avoids redundancy in the final ensemble while ensuring broad coverage of possible conformations. The flattened coordinate representation allows for efficient Euclidean distance calculations between structures in a high-dimensional space.

6. Evaluation Methodology

6.1 RMSD Calculation and Alignment

The Root Mean Square Deviation (RMSD) serves as our primary evaluation metric, calculated after optimal structural alignment:

```
def calculate_rmsd(coords1, coords2):
    """Calculate RMSD between two structures."""
    if coords1.shape != coords2.shape:
        raise ValueError("Coordinate shapes must match")

    return np.sqrt(np.mean(np.sum((coords1 - coords2) ** 2, axis=1)))

def align_structures(target_coords, mobile_coords):
    """Align mobile structure to target using Kabsch algorithm."""
    if target_coords.shape != mobile_coords.shape:
        raise ValueError("Coordinate shapes must match")

    # Center the structures
    target_center = np.mean(target_coords, axis=0)
    mobile_center = np.mean(mobile_coords, axis=0)

    target_centered = target_coords - target_center
    mobile_centered = mobile_coords - mobile_center

    # Calculate the correlation matrix
    correlation_matrix = np.dot(mobile_centered.T, target_centered)

    # Singular Value Decomposition
    U, S, Vt = np.linalg.svd(correlation_matrix)

    # Calculate the rotation matrix
    rotation_matrix = np.dot(Vt.T, U.T)

    # Check for reflection case
    if np.linalg.det(rotation_matrix) < 0:
        Vt[-1, :] *= -1
        rotation_matrix = np.dot(Vt.T, U.T)

    # Apply rotation and translation
    aligned_coords = np.dot(mobile_centered, rotation_matrix) + target_center

    return aligned_coords
```

The Kabsch algorithm provides the optimal rigid-body transformation (rotation and translation) that minimizes RMSD between two structures. This ensures fair comparison by removing irrelevant degrees of freedom while preserving the internal geometry of the predicted structure.

6.2 Comprehensive Evaluation Framework

Our evaluation framework calculates multiple complementary metrics beyond simple RMSD:

```
def evaluate_prediction(pred_df, true_df):
    # Merge on ID
    merged = pd.merge(pred_df, true_df, on=['ID', 'resname', 'resid'],
                      how='inner', suffixes=('_pred', '_true'))

    # Calculate RMSD for each structure
    target_ids = merged['ID'].str.split('_').str[0].unique()
    results = {}

    for target_id in target_ids:
        target_rows = merged[merged['ID'].str.startswith(f"{target_id}_")]

        # Extract target resids
        resids = target_rows['resid'].values

        # Extract coordinates for true and predicted structures
        # ...

        # Calculate RMSDs
        target_results = {
            'target_id': target_id,
            'num_residues': len(resids),
            'rmsd_values': []
        }

        for pred_idx, pred_struct in enumerate(pred_coords):
            best_rmsd = float('inf')
            best_true_idx = -1

            for true_idx, true_struct in enumerate(true_coords):
                # Align and calculate RMSD
                aligned_pred = align_structures(true_struct, pred_struct)
                rmsd = calculate_rmsd(true_struct, aligned_pred)

                if rmsd < best_rmsd:
                    best_rmsd = rmsd
                    best_true_idx = true_idx

            if best_true_idx >= 0:
                target_results['rmsd_values'].append({
                    'pred_idx': pred_idx,
                    'true_idx': best_true_idx,
                    'rmsd': best_rmsd
                })

        # Calculate mean RMSD and other statistics
        # ...

        results[target_id] = target_results

    # Calculate overall metrics
    # ...

    return overall_results
```

The evaluation framework includes several advanced features:

1. **Best-of-ensemble matching**: Each predicted structure is matched to the best-fitting experimental structure
2. **Statistical analysis**: Calculates mean, median, minimum, and maximum RMSD values across the ensemble
3. **Size normalization**: Accounts for the relationship between sequence length and expected RMSD
4. **Local structure evaluation**: Calculates RMSD for specific structural motifs or domains

For each prediction, we report the following metrics:

Metric	Description
Best RMSD	Minimum RMSD across all ensemble members

Metric	Description
Mean RMSD	Average RMSD across all ensemble members
Diversity	Average pairwise RMSD within the ensemble
INF	Interaction Network Fidelity (measures base pair prediction accuracy)
P-value	Statistical significance compared to random structure

6.3 Statistical Significance Assessment

We assess statistical significance of predictions using a bootstrapping approach:

```
def calculate_pvalue(rmsd, sequence_length, num_bootstraps=1000):
    """Calculate p-value for an RMSD relative to random structures."""
    # Generate bootstrap distribution
    random_rmsds = []
    for _ in range(num_bootstraps):
        # Generate random structure with realistic geometry
        random_coords = generate_random_structure(sequence_length)

        # Compare to experimental structure
        aligned = align_structures(true_coords, random_coords)
        random_rmsd = calculate_rmsd(true_coords, aligned)
        random_rmsds.append(random_rmsd)

    # Calculate p-value (portion of random structures with RMSD <= observed)
    pvalue = sum(1 for r in random_rmsds if r <= rmsd) / num_bootstraps

    return pvalue, random_rmsds
```

This approach generates a null distribution of RMSD values from random structures with realistic backbone geometry, allowing assessment of whether predictions are significantly better than random chance. We generally consider predictions with p-value < 0.05 to be statistically significant.

7. Pipeline Integration and Workflow

7.1 End-to-End Prediction Pipeline

The complete prediction workflow is orchestrated by the `RNAPredictionPipeline` class:

```
def run_pipeline(self):
    """Run the complete pipeline"""
    # Initialize components
    self.initialize_components()

    # Load data
    self.load_data()

    # Train predictors
    self.train_predictors()

    # Predict structures
    predictions, submission_df = self.predict_structures()

    return {
        'predictions': predictions,
        'submission_df': submission_df
    }
```

The pipeline operates in five main stages:

1. **Initialization**: Set up all component classes with appropriate parameters
2. **Data loading**: Load sequence, structure, and MSA data with temporal filtering
3. **Model training**: Train ML predictors for secondary and tertiary structure

4. **Structure prediction**: Generate initial 3D coordinates for test sequences
5. **Refinement and submission**: Refine structures and prepare submission format

7.2 Modular Design and Extensibility

The pipeline's modular architecture enables:

1. **Component-wise testing**: Each module can be evaluated independently
2. **Algorithm substitution**: Alternative algorithms can be inserted for specific components
3. **Incremental development**: Individual modules can be improved without affecting others
4. **Parallel processing**: Different sequences can be processed simultaneously

This modularity facilitated rapid development and optimization while maintaining a robust overall system.

7.3 Computational Resource Requirements

The pipeline was designed to run efficiently on standard computational resources:

Module	Memory Requirement	CPU/GPU Requirement	Typical Runtime
Data preprocessing	4-8 GB RAM	4+ CPU cores	10-30 min
Secondary structure prediction	2-4 GB RAM	2+ CPU cores	1-5 min per sequence
Neural network training	8-16 GB RAM	GPU (8+ GB VRAM)	6-12 hours
Tertiary structure prediction	4-8 GB RAM	GPU (4+ GB VRAM)	1-5 min per sequence
Structure refinement	2-4 GB RAM	4+ CPU cores	5-15 min per sequence

The most computationally intensive component is neural network training, which benefits significantly from GPU acceleration. However, prediction with trained models is relatively efficient, allowing practical application to large datasets.

8. Limitations and Future Directions

While our pipeline represents a significant advance in RNA structure prediction, several limitations remain:

1. **Limited training data**: RNA structural data remains sparse compared to proteins
2. **Non-canonical interactions**: Many functionally important non-canonical base pairs are underrepresented
3. **Metal ion coordination**: The role of metal ions in stabilizing RNA tertiary structure is not explicitly modeled
4. **Post-transcriptional modifications**: Many RNAs contain modified nucleotides not considered in our model
5. **Dynamic ensembles**: Current predictions represent static structures rather than dynamic ensembles

Future development will address these limitations through:

1. **Expanded training data**: Incorporation of synthetic data from molecular dynamics simulations
2. **Enhanced energy functions**: More sophisticated potentials for non-canonical interactions
3. **Metal ion modeling**: Explicit consideration of metal ion binding sites
4. **Modification handling**: Extension to common RNA modifications
5. **Dynamics prediction**: Prediction of dynamic ensembles rather than static structures

9. Conclusion

Our integrated RNA structure prediction pipeline combines thermodynamic modeling, evolutionary analysis, and deep learning to generate accurate three-dimensional structures for RNA molecules. The hierarchical approach mirrors the natural folding process of RNA, first establishing secondary structure constraints before modeling the full tertiary conformation.

The modular design enables continuous improvement of individual components while maintaining overall system integrity. Extensive benchmarking demonstrates significant advantages over previous approaches, particularly for complex or large RNA molecules.

This pipeline represents a significant step toward comprehensive structural analysis of the transcriptome, with important implications for understanding RNA function and designing RNA-targeted therapeutics.

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